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FURTHER STUDIES ON CONTROL OF RNA SYNTHESIS BY A MUTANT OF EASTERN EQUINE ENCEPHALITIS VIRUS

Eugene Zebovitz
Arthur Brown

DECEMBER 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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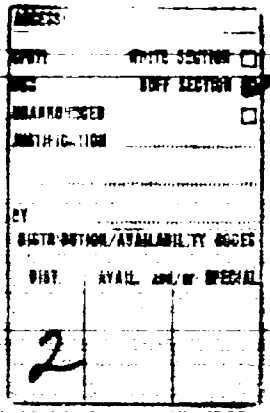

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FURTHER STUDIES ON CONTROL OF RNA SYNTHESIS BY A MUTANT OF EASTERN EQUINE ENCEPHALITIS VIRUS

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Project 1B061102B71A

December 1969

ABSTRACT

A temperature-sensitive mutant derived from eastern equine encephalitis (EEE) virus was found to induce the synthesis of a large amount of viral RNA in infected chick embryo (CE) cells. The mutant, designated Ets-4, synthesized two to four times more RNA than did the parent; however, its yield of mature virus was 0.5 to 1.0 $\log_{10}$ units less than that of EEE virus.

Simultaneous infection of CE cells with parent and mutant viruses reduced the amount of RNA synthesized by Ets-4 to a level that approximated that of EEE alone. Ets-4 virus was apparently defective with respect to the control mechanisms involved in RNA synthesis, and the appearance of large amounts of viral RNA of reduced biological activity was the result of this loss of control. The presence of EEE virus in cells infected with Ets-4 appeared to provide the mechanism to limit the production of RNA by the mutant to normal levels.

The viral RNA polymerase activity induced in CE cells infected with Ets-4 virus was higher than with the parent virus. The presence of EEE virus in Ets-4 - infected cells, however, reduced the amount of RNA polymerase synthesized. This observation suggested that EEE must exert its influence at some time before or during the synthesis of viral RNA polymerase.

It appeared likely that EEE virus acts upon Ets-4 RNA synthesis at some point between the penetration of the cell by the virus and the synthesis of the viral RNA polymerase. EEE or Ets-4 viral RNA labeled with H^3-uridine was used to infect CE cells, which at intervals after infection were disrupted and the radioactivity associated with the microsomal fraction was determined. The radioactivity arising from Ets-4 RNA became associated with the cellular microsomes in greater amounts than did that of the parent virus, indicating that this RNA may be bound more efficiently to the microsomal fraction. Experiments are in progress to determine the effect of EEE virus infection upon the binding of Ets-4 RNA to the microsomal fraction of CE cells.

I. INTRODUCTION*

Several years ago we isolated a temperature-sensitive mutant of eastern equine encephalitis (EEE) virus that exhibited an unusual amount of RNA synthesis. The properties of this virus have been described in two previous reports.** The mutant virus, designated Ets-4, was found to induce, in infected chick embryo (CE) cells, the synthesis of two to four times more viral RNA than the parent, but the yield of mature virus was generally 0.5 to 1.0 $\log_{10}$ less. Three types of viral RNA were detected for both the parent and the mutant viruses, the 45S infectious RNA, a 27S or intermediate RNA, and the double-stranded 20S RNA; however, the amount of each RNA type made by Ets-4 was substantially greater. The 45S infectious RNA made by Ets-4 was calculated to have one-tenth the specific infectivity of EEE virus. On the basis of these data it was concluded that the Ets-4 mutant was defective with respect to the control mechanisms involved in its RNA synthesis in that it produced excessive amounts of viral RNA of reduced biological activity.

This report describes our efforts to determine the nature of the control mechanisms involved in the RNA synthesis of the mutant and the parent viruses.

II. RESULTS

We can consider the unusual amount of RNA synthesized by Ets-4 as indicating a loss of control by the mutant of its own RNA synthesis. The parent virus may possess a mechanism that limits the production of viral RNA. If this is true, then it may be possible to restore control of RNA synthesis to normal levels by superinfecting CE cells already infected with Ets-4 virus with EEE virus. The experimental results we obtained are shown in Figure 1.

The sedimentation pattern of RNA extracted from CE cells infected simultaneously with EEE and Ets-4 is shown in Figure 1. The multiplicity of infection for both viruses was adjusted to 10 pfu/cell. The viruses

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* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

** Zebovitz, E.; Brown, A. January 1968. Pattern of viral RNA synthesis in a temperature-sensitive mutant of eastern equine encephalitis virus, (Technical Manuscript 432). Virus and Rickettsia Division, Fort Detrick, Frederick, Maryland.
Zebovitz, E.; Brown, A. February 1969. Control of RNA synthesis in eastern equine encephalitis viruses, (Technical Manuscript 506). Virus and Rickettsia Division, Fort Detrick, Frederick, Maryland.

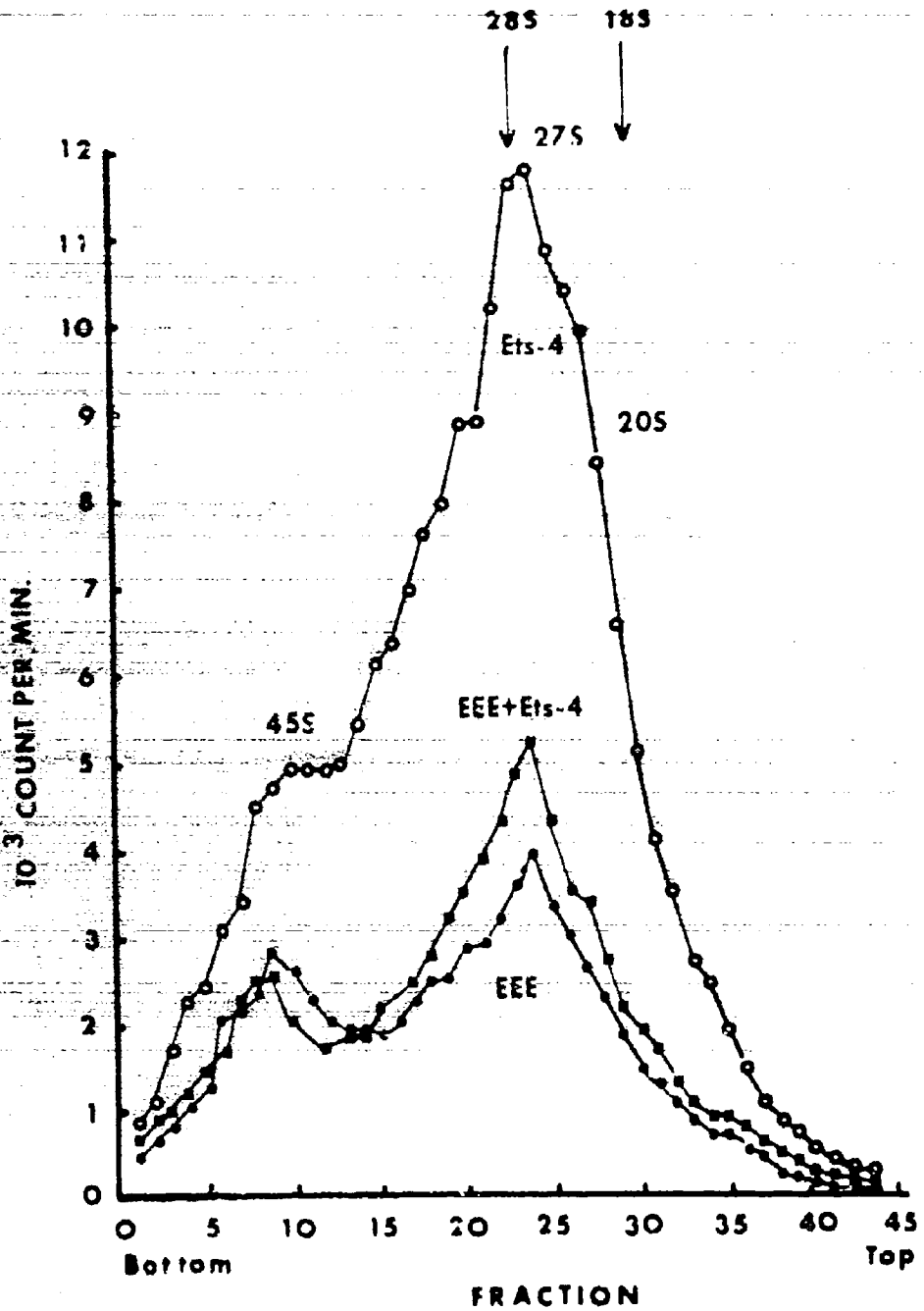

FIGURE 1. Effect of Double Infection of Chick Embryo Cells with EEE and Ets-4 Viruses Upon the Viral RNA Sedimentation in Sucrose Gradients.

were allowed to replicate together for 6 hours at 37 C. The upper curve is the sedimentation pattern of RNA synthesized by CE cells infected with Ets-4 virus alone. The lower curve is that of EEE virus alone. The RNA pattern of the doubly infected cells is the middle curve. The sedimentation pattern of RNA extracted from doubly infected cells was similar to those observed for singly infected cultures, but the amount of RNA made in these cells approximated that of EEE virus grown alone. These data indicate that Ets-4 RNA synthesis was strongly influenced by the presence of EEE virus growing in the same cell.

The time of superinfection with EEE virus was important (Fig. 2). EEE virus could reduce Ets-4 RNA synthesis to normal levels if it was added up to 1 hour after infection with Ets-4, but if it was added at 2 hours or later, it could not lower the amount of Ets-4 RNA synthesized. These results suggest that EEE virus could influence RNA synthesis of Ets-4 virus only during the early stages of Ets-4 infection of CE cells.

To compare further the RNA synthesis of EEE and Ets-4 viruses, the viral RNA polymerase induced in infected cells by both viruses was studied. If the viral RNA polymerase of Ets-4 is responsible for the formation of excessive amounts of viral RNA, then it is possible that EEE may in some way influence the synthesis or activity of the enzyme. The effect of double infection of CE cells upon viral RNA polymerase activity was determined next.

CE cells were infected with each virus singly or simultaneously and overlaid with a maintenance medium containing 1 μg of actinomycin D per ml and incubated at 37 C. At 2-hour intervals the cells from five 60-mm plates were washed and pooled, pelleted by centrifugation, and frozen rapidly. The polymerase activity was determined within 1 day. The cellular microsomal fraction used as the enzyme source in the polymerase test was obtained by lysing the cells in a hypotonic salt solution and subjecting the suspension to ten strokes with a Dounce homogenizer.* The cell debris was removed by centrifugation at 600 x g and the microsomal fraction was sedimented from the supernatant cell extract by centrifugation at 10,000 x g for 10 minutes. The microsomal pellet was assayed immediately for viral RNA polymerase activity. A reaction mixture containing tritiated guanosine triphosphate, the other unlabeled nucleotide bases, and actinomycin D was incubated with the cellular microsomal fraction for 1 hour at 37 C. The acid-precipitable radioactivity was determined by a method described by Plagemann.**

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* Kontes Glass Co., Vineland, N.J.

** Plagemann, P.G.W. 1968. Reversible inhibition of induction of mengovirus RNA polymerase and of virus maturation in Novikoff rat hepatoma cells by phenethyl alcohol. Virology 34:319-330.

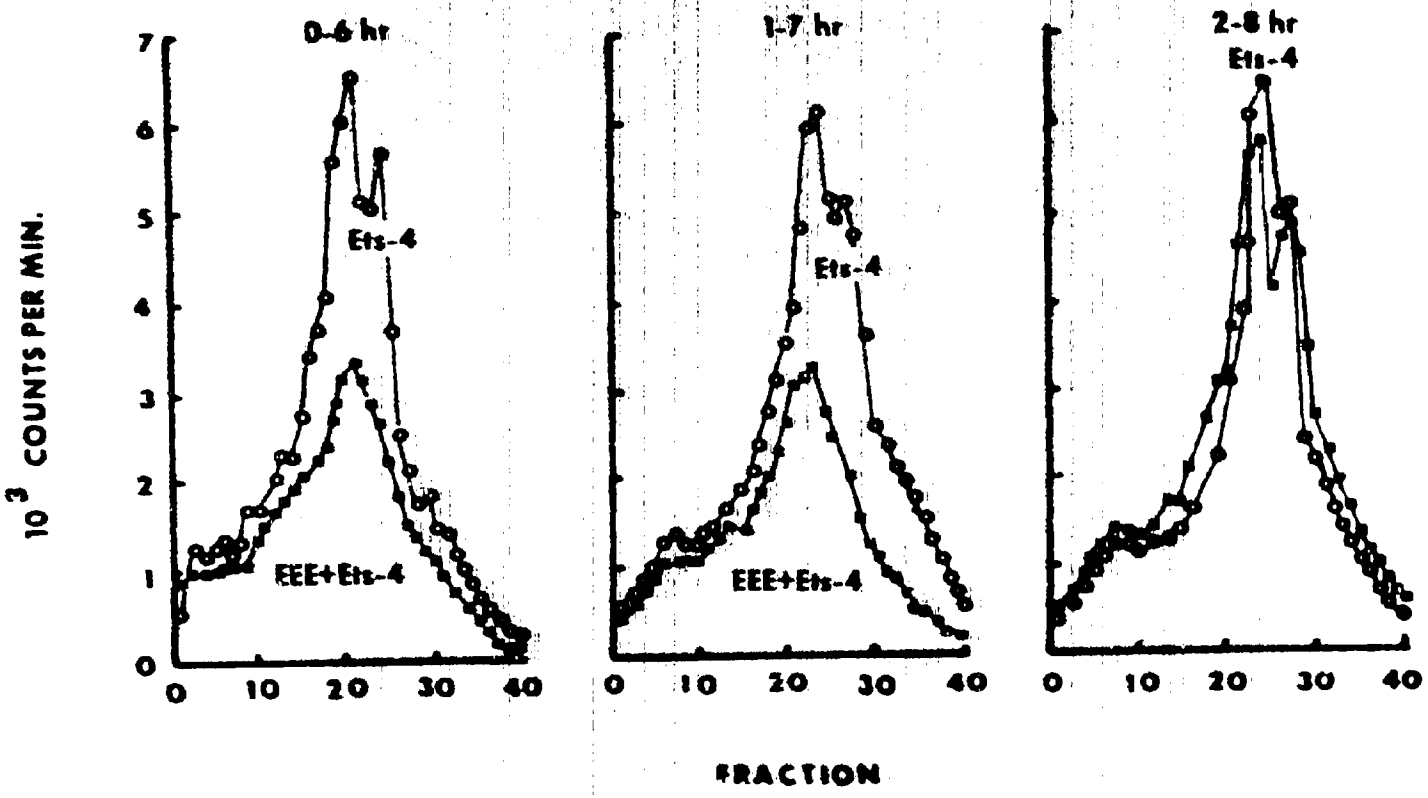

FIGURE 2. Effect of Time of Superinfection with EEE Virus Upon Ets-4 RNA Synthesis.

The rate of increase of viral RNA polymerase activity in cells infected singly with EEE or Ets-4 and cells doubly infected with both EEE and Ets-4 is shown in Figure 3. The lower curve (control) represents the endogenous polymerase activity in uninfected CE cells. The low level of activity of the control cells demonstrates that the observed RNA polymerase activity in the infected cells was specifically induced by the presence of virus in these cells. In this experiment the RNA polymerase activity of cells infected with Ets-4 virus alone had approximately four times the RNA polymerase activity after 6 hours' growth than that for EEE alone. The enzyme activity of cells infected simultaneously with EEE and Ets-4 viruses was markedly depressed and approached that of EEE alone.

It is clear from this experiment that the presence of EEE virus with Ets-4 in some manner affects the synthesis or the activity of viral RNA polymerase. Earlier experiments showed that EEE influenced Ets-4 RNA synthesis only if it was present during the first hour of infection, indicating that EEE must act very early in the infection. If the activity of Ets-4 RNA polymerase is already affected by EEE virus (Fig. 3), then it seems reasonable to assume that EEE virus must exert its influence before the synthesis of viral RNA polymerase. Because RNA polymerase is believed to be the very first protein synthesized in a virus infection, then it must be considered likely that the effect of EEE virus on Ets-4 synthesis occurs before the initiation of protein synthesis, and that a component of the virus particle, either protein or RNA, may be involved.

Detailed investigations of the steps involved between the entry of the virus particle into the cell and the synthesis of viral RNA are in progress. One approach to this problem was to determine the fate of the incoming viral RNA after infection of cells with purified virus containing H^3-labeled viral RNA. The virus was labeled by growing it for 18 hours on CE cells overlaid with a serum-free maintenance medium containing 5 μc of H^3-uridine per ml. The virus was harvested from the culture fluids by centrifugation and further purified by banding in a sucrose gradient. The acid-precipitable radioactivity of the purified virus preparations were determined and adjusted by dilution to approximately the same level of radioactivity. The diluted purified virus suspension served as the virus inoculum.

One-tenth ml of radioactive virus inoculum was used to infect CE cell monolayers in 60-mm plates. The virus was allowed to adsorb at 4 C for 30 minutes, the overlayers were then overlaid with maintenance medium containing actinomycin D and incubated at 37 C. At 2-hour intervals, samples of five plates each were removed from the incubator and processed to obtain the cellular microsomal fraction in the same manner described for the RNA polymerase assay. The microsomes were washed twice in a tris buffer and dissolved in 0.5 ml Triton X-100; then the radioactivity was determined in a liquid scintillation spectrometer.

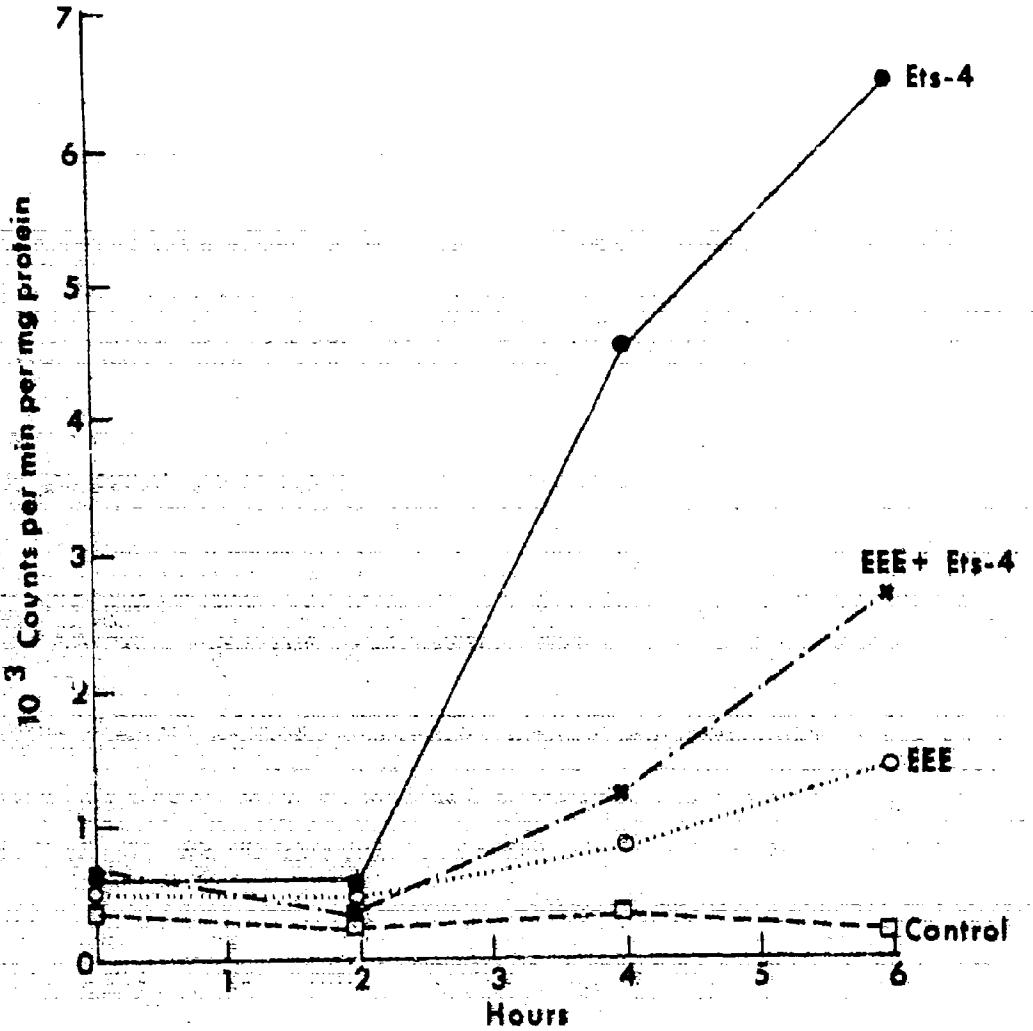

FIGURE 3. RNA Polymerase Activity of CE Cells Infected with EEE, Ets-4, and a Combination of EEE + Ets-4 Viruses.

Figure 4 shows a comparison of the radioactivity associated with the microsomal fraction after infection of CE cells with radioactive EEE and Ets-4 RNA. Because H^3-uridine was used to label the virus, it was assumed that most of the label was incorporated into the viral RNA. The radioactivity that became associated with the microsomal fraction is believed to be viral RNA that entered the cell in the intact virus particle; when the virus protein coat was removed, the RNA became associated with the microsomal fraction. If these assumptions are correct, then it appears that Ets-4 RNA tends to bind to the microsomal fraction in larger amounts than that of EEE virus. The titer of EEE virus inoculum was greater than that of Ets-4, yet the amount of radioactivity fixed to the microsomes was less. The binding of viral RNA was not inhibited in the presence of the protein inhibitor cycloheximide, which indicates that the binding can occur in the absence of protein synthesis.

These results show that there is a difference in the capacity of EEE and Ets-4 viral RNA to bind to the microsomal fraction. Ets-4 appears to be bound more efficiently, and this property of Ets-4 may account for the synthesis of large amounts of viral RNA.

The role of EEE virus in lowering the rate of Ets-4 RNA synthesis is not yet known, but it seems likely at this time that EEE virus or one of its components may block the binding of Ets-4 RNA to some replication site on the microsomal fraction.

These studies are being continued with special emphasis on the capacities of EEE and Ets-4 RNA to bind to the cellular microsomal fraction and on determining the species of RNA associated with the microsomes.

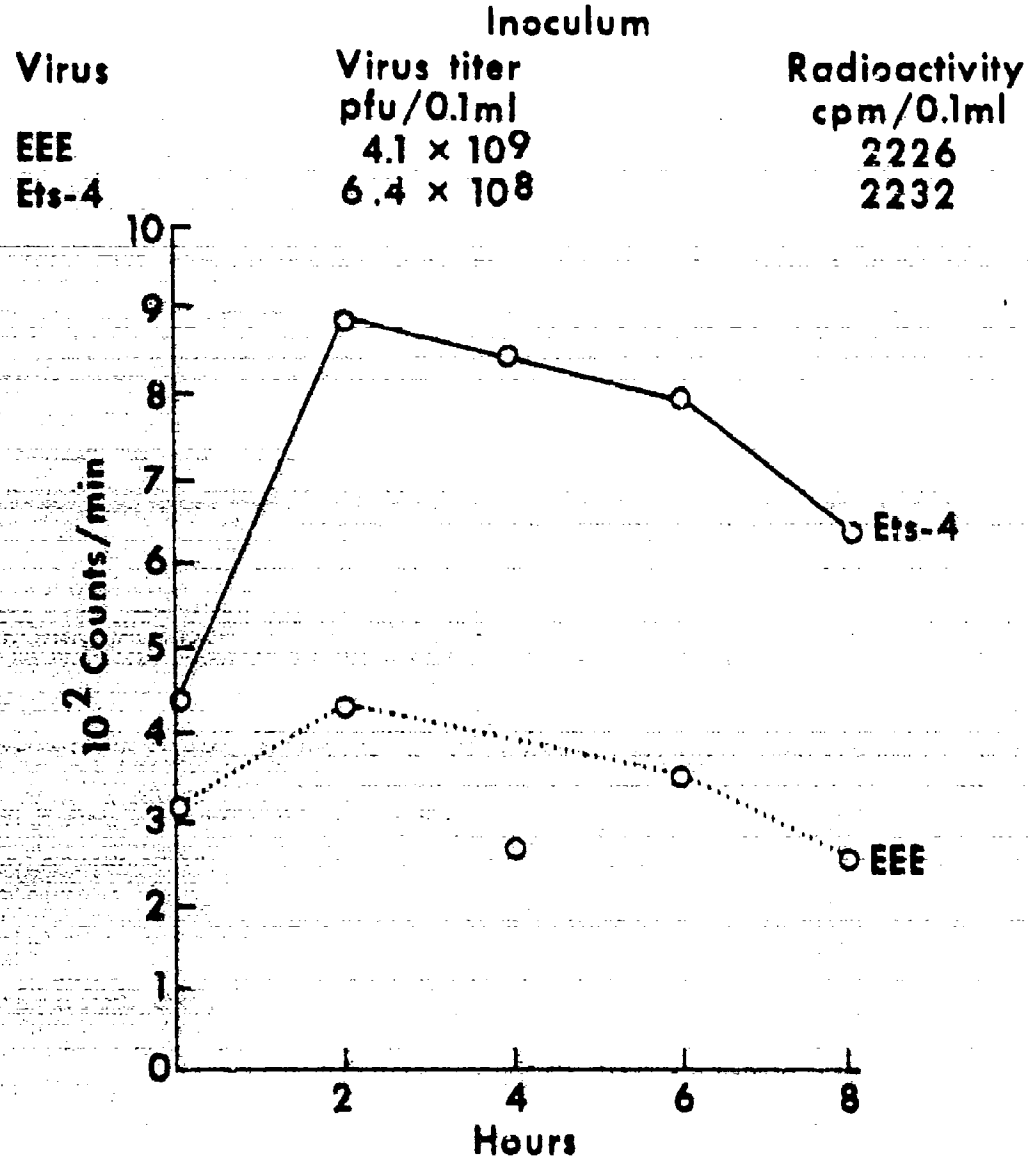

FIGURE 4. Radioactivity Associated with Microsomes Isolated from CE Cells Infected with EEE and Ets-4 Viruses with H^3-Labeled RNA.

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14. Key Words

EEE virus
Viral RNA synthesis
RNA polymerase
Temperature-sensitive viruses